

A Microbiological Profile of Symptomatic Teeth with Primary Endodontic Infections

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Abstract

The aim of this study was to evaluate the composition of the microbiota of primary endodontic infections associated with symptomatic teeth. Samples were collected by means of a #15 H-type file and 2 sterile paper points from 60 symptomatic ($n = 30$) or asymptomatic ($n = 30$) single-rooted teeth with necrotic pulp. The presence of 40 bacterial species was determined by the checkerboard DNA-DNA hybridization method. The species found in higher counts ($\times 10^5$) in symptomatic cases were *Fusobacterium nucleatum ssp. vincentii*, *Veillonella parvula*, *Treponema socranskii*, *Enterococcus faecalis*, and *Campylobacter gracilis* and in asymptomatic cases were *F. nucleatum ssp. vincentii*, *Fusobacterium nucleatum ssp. nucleatum*, *E. faecalis*, *Eubacterium saburreum*, and *Neisseria mucosa*. Total bacterial counts and counts of *Tannerella forsythia* were significant higher in symptomatic cases ($p < 0.05$), whereas levels of *Propionibacterium acnes* were reduced in this group of teeth. The data of the present investigation suggested an association between higher total bacterial counts and levels of *T. forsythia* and the presence of pain. (*J Endod* 2008;34:541–545)

Key Words

Bacteria, clinical, DNA probes, endodontics, symptoms

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The introduction of microbiological molecular diagnostic methods represented a great improvement on the identification of the microbiota related to endodontic infections. Many culture studies have shown that these infections are associated with a mixed microbiota, mainly composed by obligate anaerobes such as *Fusobacterium*, *Porphyromonas*, and *Prevotella* (1–3). The use of these new molecular techniques lead to a better comprehension of the microbial profile of primary endodontic infections because it allows the identification of some species that were underestimated in culture studies, such as *Treponema spp.* (4–8), *Filifactor alocis* (7, 9), *Tannerella forsythia* (7, 10–12), and *Enterococcus faecalis* (10, 13). One of these molecular methods, called the checkerboard DNA-DNA hybridization, allows a quantitative analysis of the bacterial species evaluated and is able to hybridize large numbers of samples against large numbers of DNA probes on a single-support membrane (14).

Primary endodontic infections may be associated to some clinical aspects and symptoms such as pain, periapical lesion, tenderness to percussion, swelling, and sinus tract. These symptoms may be consequence of an acute inflammation of the apical periodontal ligament (15) or the answer to a long-time presence of bacterial irritants causing a chronic apical lesion (16). It has been described that endodontic microbiota of asymptomatic teeth presents some differences from symptomatic teeth (17–19). Some bacterial species have been related to symptomatic teeth, such as black-pigmented (20, 21) *Prevotella* and *Peptostreptococcus* species (3, 22).

Therefore, the purpose of this study was to evaluate the composition of the microbiota of primary endodontic infections associated with symptomatic teeth using a non-culturing microbiological diagnostic method that allows a more accurate evaluation of the microbial profile.

Materials and Methods

Subject Population

The subject population was composed of 60 subjects selected from the Endodontics Clinic of three different Brazilian Dental Schools: Rio de Janeiro State University, Gama Filho University, and UNIGRANRIO University. All subjects were informed about the study's nature and after the signing of an ethics committee–approved informed consent, they were entered into the study. This work was approved by The Research Ethics Committee of Rio de Janeiro State University (#745-Research Ethics Committee/Pedro Ernesto Hospital).

Inclusion and Exclusion Criteria

To be included in the study, subjects had to have good systemic health, with at least one single-rooted tooth with necrotic pulp and radiographic evidence of bone loss, with ($n = 30$) or without the presence of pain ($n = 30$). Three of the asymptomatic and five of the symptomatic cases presented associated sinus tract. In all subjects, a cold test with Endo frost (Roeko, Langenau, Germany) was done to confirm pulpal necrosis. The presence of pain was assessed by case history and clinical periapical tests. Percussion sensitivity test was performed by tapping on the incisal or on the occlusal surface with the end of a mirror handle held parallel or perpendicular to the crown. Palpation sensitivity test was performed by a firm pressure on the mucosa overlying the apex. Exclusion criteria were the presence of periodontal pockets greater than 4 mm, pregnancy, nursing, and any systemic condition that could affect the progression of infectious disease or required antibiotic coverage for routine dental therapy. In addition,

subjects who received antibiotic or anti-inflammatory therapy in the previous 6 months were excluded. Besides, none of the sampled teeth presented either crowns or bridges.

Sample Collection

Samples were collected from each of the 60 teeth under strict aseptic conditions as described previously (10). Initially, the tooth was cleaned with pumice and isolated with rubber dam. The tooth and the rubber dam were cleaned with a solution of 3% hydrogen peroxide and then disinfected with 2.5% NaOCl solution. The coronal access was made with the use of sterile round burs without water spray. The pulp chamber and the operator field were disinfected again by using a swab soaked in 2.5% NaOCl. This solution was inactivated with sterile 5% sodium thiosulfate. Samples were collected from the root canal by means of a #15 H-type file (Dentsply/Maillefer, Ballaigues, Switzerland) with the handle cut off, with a discrete filing motion, introduced 1 mm short of apical foramen. This length was determined by means of a periapical radiograph and a metallic rule. Subsequently, 2 sterile paper points were introduced in the root canal at the same level of the file, and each left for 1 minute to soak up the tissue fluid. Both file and paper points were then transferred to Eppendorf tubes containing 150 μ L of TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.6); 0.1 mL of 0.5 mol/L NaOH was added to each tube, and the samples were frozen at -20° C until they were processed.

Microbiologic Assessment

The presence and levels of 40 bacterial species (Table 1) were determined in each sample using a modification (23) of the checkerboard DNA-DNA hybridization method described by Socransky et al. (14).

Briefly, the samples were boiled for 10 minutes and neutralized by using 0.8 mL of 5 mol/L ammonium acetate. The released DNA was then fixed in individual lanes of a positively charged nylon membrane (Amersham Biosciences, Chicago, IL) using a Minislot 30 apparatus (Immunetics, Cambridge, MA). A Miniblotter 45 (Immunetics) device was used to hybridize 40 digoxigenin-labeled whole genomic DNA probes (Table 1) perpendicularly to the lines of the clinical samples. The DNA probes were prepared using the random primer digoxigenin labeling kit

(Boehringer Mannheim, Indianapolis, IN) (24). Bound probes were detected by using a phosphatase-conjugated antibody to digoxigenin and chemiluminescence (CDP-Star Detection Reagent, Amersham Biosciences). Signals were evaluated visually by comparison with two lanes of standards on the same membrane, containing a mixture of the species evaluated at 10^5 and 10^6 cells. The signals were recorded as follows: (0) not detected, (1) $<10^5$ cells, (2) approximately 10^5 cells, (3) 10^5 to 10^6 cells, (4) approximately 10^6 cells, and (5) $>10^6$ cells. The sensitivity of this assay was adjusted to permit detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe.

Statistical Analysis

The data were expressed in prevalence (presence or absence) and mean counts ($\times 10^5$ cells) of each species. Differences between asymptomatic and symptomatic cases were sought using the Mann-Whitney *U* test for individual bacterial species. The comparison of the mean total counts of the 40 species evaluated between the two groups and among all genera evaluated was assessed using a *t* test. The level of significance was established at 5%.

Results

The population age ranged from 18 to 70 years-old (mean age = 34), and 62% of the subjects were women. In asymptomatic cases, three of them presented an associated sinus tract, 20 teeth had permanent restorations (18 were clinically and/or radiographically defective) and cavities were evident in 20. Among the symptomatic cases, 5 presented an associated sinus tract, 11 teeth had permanent restorations (nine were clinically and/or radiographically defective), and cavities were evident in 12. From these 30 cases, 15 teeth presented pain to percussion, 5 teeth presented pain to palpation, and 10 presented pain to both percussion and palpation. Each of the 40 bacterial species evaluated was present in at least 4 samples. The mean number of species found in the 60 samples was 24, ranging from 7 to 38 species per sample. In samples from symptomatic teeth, the mean number of species detected was 26 ranging from 11 to 38, and 20 ranging from 7 to 35 species in

TABLE 1. Strains Used for the Development of DNA Probes

Species	Strains	Species	Strains
<i>Actinomyces gerencseriae</i> ^a	23860	<i>Leptotrichia buccalis</i> ^a	14201
<i>Actinomyces israelii</i> ^a	12102	<i>Neisseria mucosa</i> ^a	19696
<i>Actinomyces naeslundii</i> 1 ^a	12104	<i>Peptostreptococcus micros</i> ^a	33270
<i>Actinomyces odontolyticus</i> ^a	17929	<i>Porphyromonas endodontalis</i> ^a	35406
<i>Aggregatibacter actinomycetemcomitans</i>	*	<i>Porphyromonas gingivalis</i> ^a	33277
<i>Campylobacter gracilis</i> ^a	33236	<i>Prevotella intermedia</i> ^a	25611
<i>Campylobacter rectus</i> ^a	33238	<i>Prevotella melaninogenica</i> ^a	25845
<i>Campylobacter showae</i> ^a	51146	<i>Prevotella nigrescens</i> ^a	33563
<i>Capnocytophaga gingivalis</i> ^a	33624	<i>Propionibacterium acnes</i>	**
<i>Capnocytophaga ochracea</i> ^a	33596	<i>Selenomonas noxia</i> ^a	43541
<i>Capnocytophaga sputigena</i> ^a	33612	<i>Streptococcus anginosus</i> ^a	33397
<i>Eikenella corrodens</i> ^a	23834	<i>Streptococcus constellatus</i> ^a	27823
<i>Enterococcus faecalis</i> ^a	29212	<i>Streptococcus gordonii</i> ^a	10558
<i>Eubacterium nodatum</i> ^a	33099	<i>Streptococcus intermedius</i> ^a	27335
<i>Eubacterium saburreum</i> ^a	33271	<i>Streptococcus mitis</i> ^a	49456
<i>Fusobacterium nucleatum</i> ssp. <i>nucleatum</i> ^a	25586	<i>Streptococcus oralis</i> ^a	35037
<i>Fusobacterium nucleatum</i> ssp. <i>polymorphum</i> ^a	10953	<i>Tannerella forsythia</i> ^a	43037
<i>Fusobacterium nucleatum</i> ssp. <i>vicentii</i> ^a	49256	<i>Treponema denticola</i> ^b	B1
<i>Fusobacterium periodonticum</i> ^a	33693	<i>Treponema socranskii</i> ^b	S1
<i>Gemella morbillorum</i> ^a	27824	<i>Veillonella parvula</i> ^a	10790

^aATCC = American Type Culture Collection, Rockville, MD.

^bFDC, Forsyth Dental Center.

*ATCC strains 43718 and 29523.

**ATCC strains 11827 and 11828.

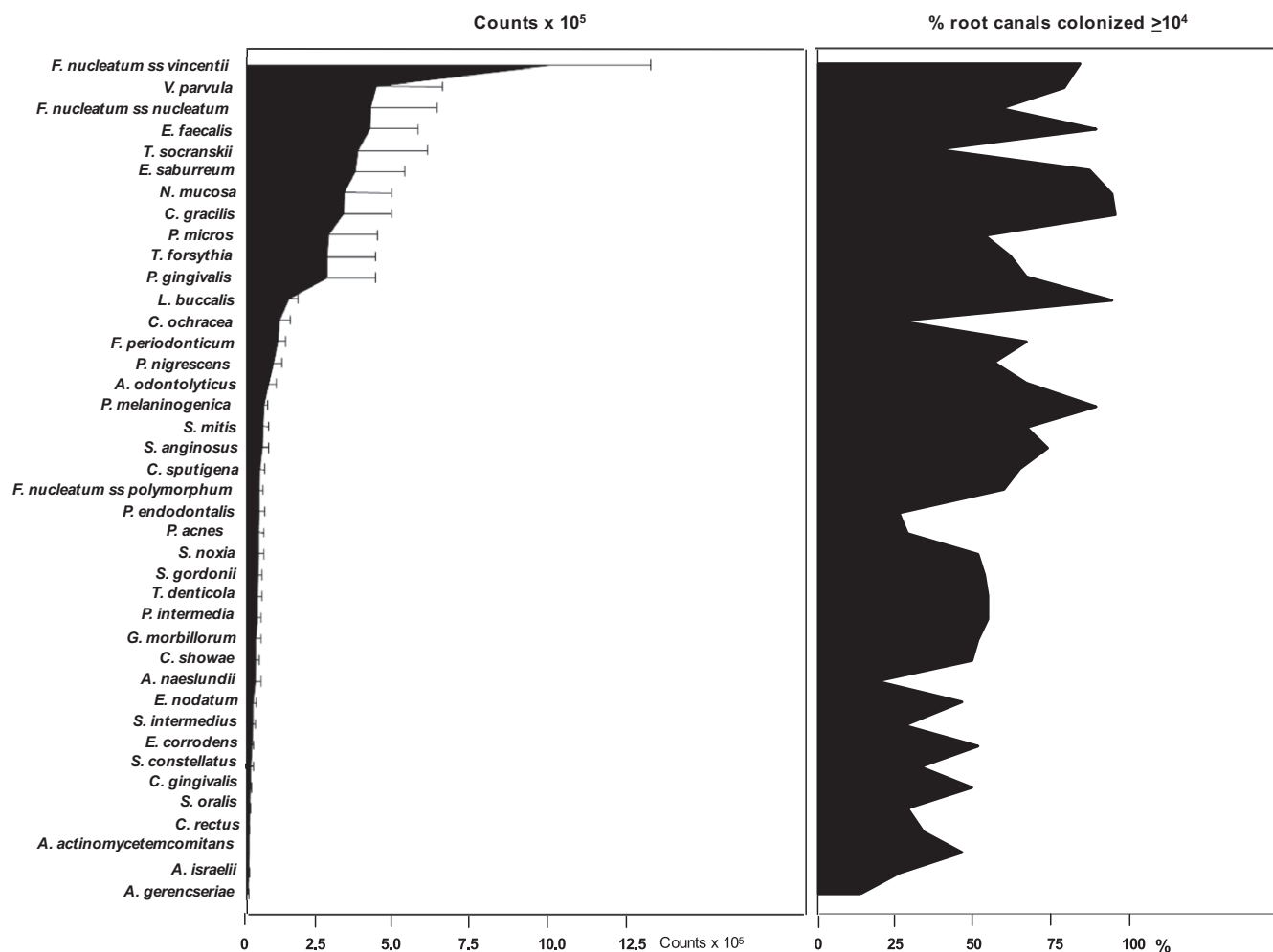


Figure 1. Mean counts ($\times 10^5 \pm \text{SEM}$, left panel) and mean prevalence (% of root canals colonized by counts $> 10^4 \pm \text{SEM}$, right panel) of 40 individual bacterial species in the 60 samples evaluated. The species are presented in descendent order according to mean counts.

asymptomatic cases. The mean number of species found in symptomatic and asymptomatic cases was statistically similar (t test, $p > 0.05$).

The mean counts ($\times 10^5 \pm \text{standard deviation}$) and the prevalence of each species evaluated in all 60 samples are presented in Figure 1. The total levels of bacteria observed in symptomatic cases was 86×10^5 (standard error = 24×10^5), and 29×10^5 (SE = 8.4×10^5) in asymptomatic cases. The difference observed between the two groups was statistically significant (t test, $p < 0.05$).

The mean levels ($\times 10^5 \pm \text{SD}$) of the individual species in symptomatic and asymptomatic cases are presented in Figure 2 in a descendent order according to the values observed in the symptomatic samples. In symptomatic cases, the bacterial species detected in highest levels were as follows: *Fusobacterium nucleatum ssp. vincentii* ($15.38 \pm 3.38 \times 10^5$), *Veillonella parvula* ($7.87 \pm 2.51 \times 10^5$), *Treponema socranskii* ($7.02 \pm 2.53 \times 10^5$), *E. faecalis* ($6.62 \pm 1.80 \times 10^5$), *Campylobacter gracilis* ($5.55 \pm 1.80 \times 10^5$), *Neisseria mucosa* (5.41 ± 1.81), and *Eubacterium saburreum* (5.35 ± 1.84). In the asymptomatic cases, the species detected in highest levels were as follows: *F. nucleatum ssp. vincentii* (5.59 ± 2.92), *F. nucleatum ssp. nucleatum* (3.80 ± 2.10), *E. faecalis* (2.26 ± 1.84), *E. saburreum* (2.20 ± 1.87), *N. mucosa* (1.30 ± 1.54), and *Peptostreptococcus micros* (1.22 ± 1.11). *T. forsythia* was found in significantly higher levels ($p < 0.05$) in samples from symptomatic teeth, and *Propionibacterium acnes* was found in significantly higher levels ($p <$

0.05) in samples from asymptomatic teeth. Individually, most of the species evaluated (except for *P. acnes*, *Capnocytophaga sputigena*, *Eikenella corrodens*, and *Eubacterium nodatum*) were found in higher levels in symptomatic cases. The genera *Streptococcus*, *Prevotella*, *Fusobacterium*, *Capnocytophaga*, *Campylobacter*, and *Actinomyces* were found in higher levels in symptomatic cases ($p > 0.05$, data not shown).

Discussion

The present study investigated the microbiological profile of 60 cases of primary endodontic infections of symptomatic and asymptomatic teeth using the checkerboard DNA-DNA hybridization technique. This molecular diagnostic method allows the analysis of a significant number of samples against a large number of different bacterial species (14). This advantage makes possible a solid evaluation of the bacterial diversity of the endodontic infections (5, 10, 12, 25). Sakamoto et al. (26), who have also evaluated the microbiota of symptomatic and asymptomatic teeth with endodontic infection, showed that the bacterial diversity is greater than previously described by culture methods (3, 20, 22). Based on these findings, the authors suggest that other studies should focus on microbial profiles of a larger number of endodontic samples using molecular methods, such as the checkerboard DNA-DNA hybridization. The development of these techniques represented a great

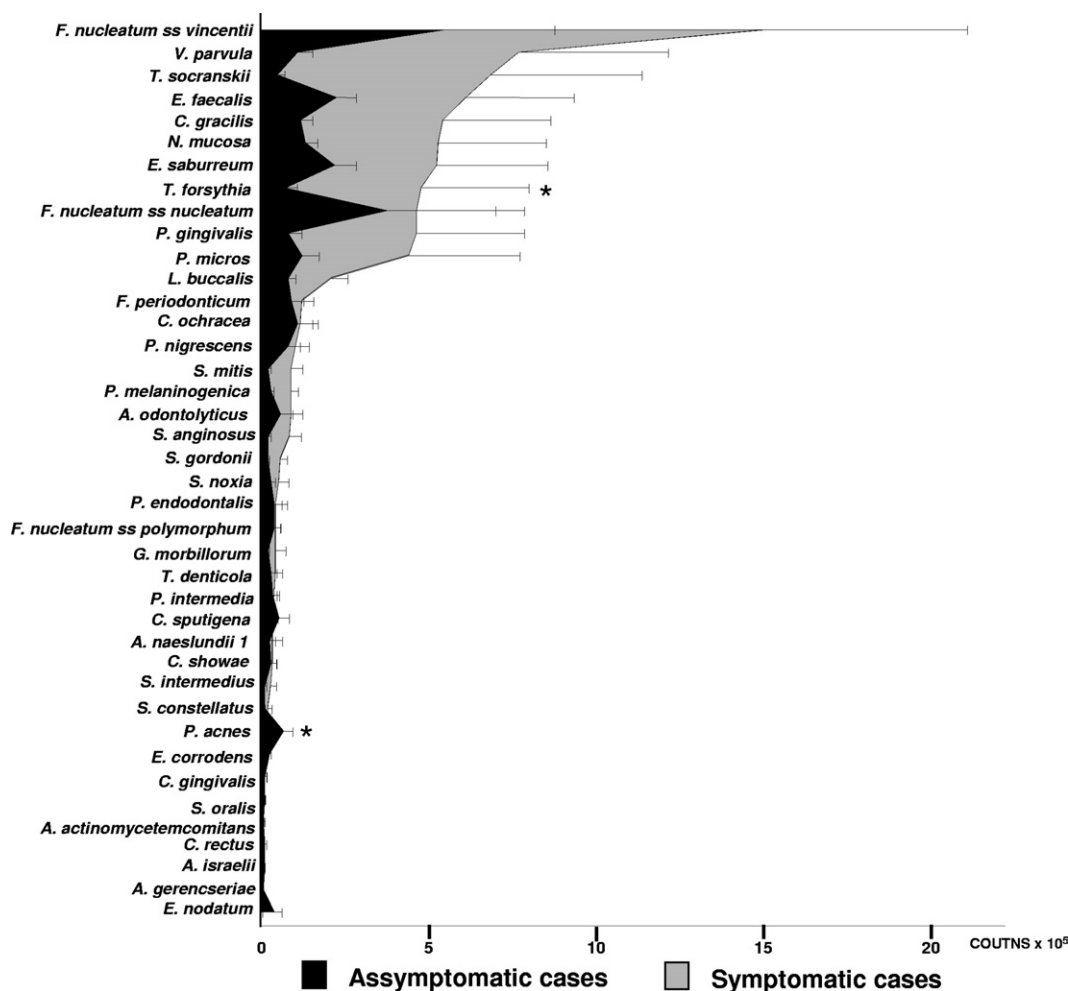


Figure 2. Counts of the 40 individual species ($\times 10^5 \pm \text{SE}$) in 30 symptomatic and 30 asymptomatic cases. The species are presented in descendent order according to mean counts of the symptomatic samples (* $p < 0.05$, Mann-Whitney U test).

improvement in this scenario because they are not dependent on the microbial viability for their detection.

The difficulties in cultivating some of the putative endodontic pathogens have delayed the better comprehension of the endodontic microbiota composition as well as possible associations between specific species and clinical signs and symptoms. It has been suggested that the endodontic microbiota can differ according to the type of periapical disease present (27). Some authors correlate cases of acute periapical lesions (1, 3, 28–30) with the presence of gram-negative species such as *Prevotella*, *Porphyromonas*, and *Fusobacterium*. Although some articles have aimed to find some relationship between the presences of pain with any specific bacterial species, this was normally done comparing chronic lesions to acute lesions (5, 8, 11, 19, 26, 31, 32). Our study aimed to compare the microbial composition of symptomatic and asymptomatic cases in chronic lesions. In the present study, the total bacterial counts were significantly higher ($p < 0.05$) in symptomatic cases. Our finding is in line with Siqueira (27) who suggested that the levels of bacterial species found in root canals may be an important indicator of the pathogenicity of this infection. Jung et al. (4) suggested that the degree of severity of an endodontic infection is related not merely to the presence of pathogens but to the numbers of those organisms in the infected site. In addition, Siqueira et al. (19), using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis of endodontic microbiota, found differences in

predominant bacterial composition between asymptomatic and symptomatic cases. The authors observed that the mean number of bands detected in the 16S rDNA community profile was 12.1 ± 9.4 for symptomatic samples and 6.7 ± 2.7 for asymptomatic ones. They suggested that the structure of the bacterial community may play a role in the development of symptoms. To assess these aspects, the checkerboard DNA-DNA hybridization method used in this study has a clear-cut advantage of allowing the identification and quantification of a large number of bacterial species found in the infected tooth (14, 23). Although the PCR technique is pointed as one of the most sensitive of the molecular diagnostic methods, it does not allow microbial quantification. Only real-time PCR permits this kind of analysis, but the high cost of this technique limits its utilization to few samples and bacterial species (33).

The endodontic microbial composition of asymptomatic and symptomatic teeth did not present striking divergences (Fig. 2). However, few important differences could be observed between the profiles of colonization of the two groups. A significant higher mean level of *T. forsythia* ($p < 0.05$) was detected in symptomatic cases. These results are in line with the reports of Siqueira et al. (5), Sassone et al. (10), and Souza et al. (12) who also described, by checkerboard DNA-DNA hybridization, a high prevalence and levels of *T. forsythia* in primary endodontic infections. Siqueira and Rôças (11) and Conrads et al. (34) detected, by PCR, the presence of *T. forsythia* in dental root canal infections and suggested the possible role of this species in the etiology

and pathogenesis of endodontic lesions. However, Rôças et al. (31), evaluating the presence of periodontal pathogens from the “red complex” (*Treponema denticola*, *Porphyromonas gingivalis*, and *T. forsythia*) in endodontic infections did not find any correlation of clinical signs and symptoms and the presence of *T. forsythia*. Gomes et al. (21) found an association of the “red complex” species with tenderness to percussion and pain on palpation. However, differently from our results, no specific correlation of *T. forsythia* and presence of pain was reported. Because several authors have been suggesting an association between the presence/levels of *T. forsythia* and root canal infection or its specific symptoms (eg, pain), other studies using cloning and sequencing of the 16S rRNA gene could be useful to clarify the role of this microorganism, or specific clonotypes, in the etiopathogenesis of this infection. Even though not statistically significant, much higher mean levels of *P. gingivalis* and *T. socranskii* were detected in symptomatic cases comparing with asymptomatic cases in the present investigation. These two species are gram-negative, strict anaerobes and related to the pathogenesis of periodontal diseases (35). *P. gingivalis* was described as the most proteolytic and pathogenic species among black-pigmented microorganisms (20). This species was found in 30% of cases by PCR (“red complex”) and in approximately 20% of cases by checkerboard DNA-DNA hybridization (5) in primary endodontic infections. In agreement with our results, *P. gingivalis* was found by Sassone et al. (10) and Souza et al. (12) in high prevalence and levels in infected root canals, using checkerboard DNA-DNA hybridization. Rôças et al. (32) recommended that *T. socranskii* should be included in the set of putative endodontic pathogens and Siqueira and Rôças (8) suggested that this species can be involved in the pathogenesis of different forms of periradicular lesions. It could be speculated that the high levels of these three pathogens detected in symptomatic root canals in the present investigation might help to maintain the presence of pain. The data of the present investigation suggested an association between higher total bacterial counts and levels of *T. forsythia* and the presence of pain.

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